JOINT INVENTORS

APPLICATION FOR
UNITED STATES LETTERS PATENT

SPECIFICATION

TO ALL WHOM IT MAY CONCERN:

Be it known that we, Adrian Auf Der Maur, a citizen of Switzerland, residing at Wehntalerstrasse 1, CH-8057 Zürich, Switzerland, and Alcide Barberis, a citizen of Switzerland, residing at Allenmoosstrasse 64, CH-8057, Zürich, Switzerland, and Dominik Escher, a citizen of Switzerland, residing at Rütistrasse 58, CH-8032, Zürich, Switzerland, have invented new and useful Intrabodies With Defined Framework That is Stable in a Reducing Environment and Applications Thereof, of which the following is a specification.

Intrabodies with d fined framework that is stable in a reducing environment and applications th reof

Cross References to Related Applications

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This application claims the priority of PCT patent application IB99/02054, filed December 28, 1999 the disclosure of which is incorporated herein by reference in its entirety.

10

Technical Field

The present invention concerns single chain fusions of variable regions of heavy and light chains of an antibody (scFv), in particular such scFv expressed

15 within a cell (intrabodies) with a defined, stable, framework.

Background Art

20 Antibodies are preferred tools for biochemical and molecular biology research, diagnostics and medical applications due to their high affinity and specificity to the antigen and due to their relatively high stability in vitro and in vivo. Antibodies are made of two

25 heavy and two light chains, which contain the variable regions at their N-termini and which are linked by disulfide bridges. Single chain antibodies have been engineered by linking fragments of the variable heavy and light chain regions (scFv). Each variable domain contains

30 three complementary determining regions (CDR) embedded in a framework. These CDRs are responsible for the interaction with the antigen. Each variable heavy and light region contains an intradomain disulfide bridge, which was reported to be critical for stability of the single chain

35 antibody (Biocca *et al.*, 1995; Derman *et al.*, 1993).

The most commonly used technique to identify single chain antibodies which bind specific epitopes is

The most commonly used technique to identify
single chain antibodies which bind specific epitopes is
by phage display and variations thereof (for review see
Hoogenboom *et al.*, 1998). This screening system has major
5   advantages over conventional techniques like immunization
or hybridoma technique, namely that it can uncover mono-
clonal single chain antibodies within a relatively short
time.

Single chain antibodies expressed within the
10   cell (e.g. cytoplasm or nucleus) are called intrabodies.
Due to the reducing environment within the cell, disul-
fide bridges, believed to be critical for antibody sta-
bility, are not formed. Thus, it was initially believed
that applications of intrabodies are not suitable. But
15   several cases are described showing the feasibility of
intrabodies (Beerli *et al.*, 1994; Biocca *et al.*, 1994;
Duan *et al.*, 1994; Gargano and Cattaneo, 1997; Greenman
et al., 1996; Martineau *et al.*, 1998; Mhashilkar *et al.*,
1995; Tavladoraki *et al.*, 1993). In these cases, in-
20   trabodies work by e.g. blocking the cytoplasmic antigen
and therefore inhibiting its biological activity.

Up to now, intrabodies were most of the time
derived from monoclonal antibodies which were first se-
lected with classical techniques (e.g. phage display) and
25   subsequently tested for their biological activity as in-
trabodies within the cell (Visintin *et al.*, 1999). Al-
though successful intrabodies are described (see above),
it is today completely unpredictable whether such an in-
trabody is functional within the cell (for reviews see
30   Cattaneo, 1998; Cattaneo and Biocca, 1999). The reasons
are most probably the different environments: Phage dis-
play and other classical techniques are performed under
oxidizing conditions, therefore disulfide bridges are
formed, whereas intrabodies must function in reducing
35   conditions. This reducing environment can lead to insuf-
ficient solubility of the intrabody and hence they form
non-functional aggregates. The solubility of an intrabody

can be modified by either changes in the framework
(Knappik and Pluckthun, 1995) or the CDRs (Kipriyanov *et
al.*, 1997; Ulrich *et al.*, 1995).

5   However, the hitherto known systems are lim-
ited with regard to their application to detect intracel-
lular targets. Therefore, it is a growing need to have a
reliable technology and system to screen directly for in-
trabodies specific for an antigen.

10   In WO 99/36569, Wittrup *et al.* describe a
method to display proteins and scFv on the cell wall of
yeast by using a yeast endogenous protein fragment de-
rived from Aga2p for localization on the cell wall. Li-
braries of proteins and scFv can be screened interacting
with other proteins. Other related systems are described
15 in EP 0 407 259 (Boquet *et al.*, 1991). These systems are
comparable to the phage display screening where the pro-
tein or peptide library is also presented on the surface.
However, these techniques cannot be used for intracellu-
lar screenings to identify intrabodies.

20   The patent document JP 11000174 (Kyoko *et
al.*, 1999) describes the use of yeast Pichia pastoris for
high level expression and secretion of antibody Fab frag-
ments. This yeast is famous for its high secretion level
and is therefore preferably used for this application.
25 The secreted antibody can be harvested by purification of
the supernatant. Furthermore, in EP 0 590 067,
WO92/22324, JP 060 30 778, US 569 8435, US 559 5889, JP
10313876 yeast is used for production of secreted pro-
teins or antibodies. EP 0 698 097 and WO 94/25591 dis-
30 close application of the production and secretion of only
the heavy chain or fragments thereof for further applica-
tions. JP 0 902 0798; JP 051 05700; and JP 050 97704 de-
scribe methods of yeast secretion to obtain hepatitis
vaccine when administered to the human body or to organ-
35 isms in general.

       It is also already known from WO 99/28502 to
use yeast for screenings of single chain antibodies. Said

application discloses the use of a DNA construct library for a single chain monoclonal antibody fusion reagent. This scFv library (therein termed sFv library) is subsequently used for screenings. However, it has now been

5   found that the stability and solubility of intrabodies can vary dramatically due to the use of a (non) specified framework. Furthermore, it could be shown that a direct correlation exists between the in vivo performance and the in vitro stability and solubility. Therefore, the use

10   of mRNA derived libraries of different scFv fragments is limited in view of the possibility to identify CDR which have a high affinity to the antigen because, although the CDRs would in principle show the required high affinity to the antigen, the corresponding framework is not solu-

15   ble enough and thus aggregates, making it impossible to select for this monoclonal scFv. Thus, there is still a need for improved antibodies, or intrabodies, respectively.

     The growing applications of scFv directed

20   against intracellular targets raise the need for reliable screening systems for intrabodies. Cytoplasmic targets of scFv are the most demanding application due to the instability of the scFv under reducing conditions and the unpredictability of the antibody stability. This stability

25   and also solubility problem can be solved by using defined frameworks, optimized for intracellular application.

Disclosure of the Invention

30   Hence, it is a general object of the present invention to provide methods for the isolation of a scFv or intrabody with defined framework that is stable and soluble in reducing environment.

     A further object of the present invention is

35   such a scFv or intrabody with defined framework that is stable and soluble in reducing environment.

Another object of the present invention is a
scFv or intrabody with defined framework that is stable
and soluble in reducing environment that is modified to
provide unique restriction sites in the CDR/framework-
5   connecting regions.

Another object of the present invention is a
library of scFvs or intrabodies with defined framework
that is stable and soluble in reducing environment, and
randomly or definedly variated CDRs.

10   Another object of the present invention is a
method for screening for antigen binding CDRs using such
scFvs or intrabodies with defined framework that is sta-
ble and soluble in reducing environment, and varied CDRs,
or a library of such scFvs or intrabodies.

15   Another object of the present invention is a
method for screening for further antigens using such scFv
or intrabodies or library, respectively.

Another object of the present invention is a
method for the identification of intrabodies with frame-
20   works that are soluble and stable under reducing condi-
tions.

The intrabodies of the present invention can
furthermore be used as agent in therapy, diagnosis or
prevention of diseases and several applications in
25   plants, such as functional knock out of a specific pro-
tein activity. The intrabodies can be used as such or as
DNA encoding such scFv.

In the scope of the present text, the terms
scFv and intrabody are largely used as synonyms, however,
30   it has to be understood that, while the stability and
solubility of the intrabodies (scFv) with defined frame-
work of the present invention in reducing environment,
e.g. within a cell, is necessary for the present inven-
tion, the application of such intrabodies (scFv) etc. is
35   not restricted to applications within a cell.

By only introducing amino acid changes within
the CDRs, such a framework according to the present in-

vention greatly increases the possibility to identify monoclonal antibodies showing the desired biological function of specific antigen recognition. Such changes in the CDRs of the scFv can be performed as random changes

5   without changing the defined framework, suitable for the cytoplasmic application of intrabodies.

In order to perform screenings of monoclonal single chain antibodies within the cell, one has to use a framework which is adapted to the redox environment of

10   the cytoplasm. Therefore a framework has to be stable and soluble enough even in the absence of disulfide bridge. Most of the scFv, however, are known not to fold into the proper structure under reducing conditions or in the absence of the cysteine, responsible for the formation of

15   intradomain disulfide bridges. Thus, in the scope of the present invention several frameworks containing identical CDRs have been compared and dramatic differences in the in vivo performance have been observed. By the inventive method the best performing framework containing the de-

20   fined CDRs for antigen recognition can be selected. This method is performed by using an intrabody to a known antigen as starting material. The linker used to connect the variable regions of heavy and light chain is not critical. It must, however, provide sufficient solubility

25   and flexibility to ensure suitable contact and folding for an interaction between CDRs and antigen. Suitable linkers have a typical length of about 5-60 amino acids, usual regular series of glycine and in order to enhance solubility 1 to 3 serine.

30

Such an inventive method for the isolation of an scFv with defined framework that is stable and soluble in a reducing environment is defined by the following steps:

35   a) a scFv library with varied frameworks and constant CDRs is generated by mutation of at least one framework encoding region of DNA sequence of a scFv to a

known antigen and by introduction of such mutations into
suitable expression vectors,

       b) host cells able to express a specific
known antigen and only surviving in the presence of anti-
5   gen-scFv-interaction are transformed with said scFv li-
brary,

       c) the thus transformed host cells are culti-
vated under conditions suitable to express the antigen
and the scFv and allowing cell survival only in the pres-
10 ence of antigen-scFv-interaction,

       d) the scFv expressed in surviving cells and
having a defined framework that is stable and soluble in
reducing environment is isolated.

       In a preferred embodiment the host cell is an
15 eukaryotic cell, in particular a yeast cell.

       By the above described method a scFv with de-
fined framework is obtainable. Such framework is also an
object of the present invention. Such a framework can be
modified to comprise specific restriction sites allowing
20 the selective exchanging of at least one CDR. Preferably
said restriction sites are located within the framework
flanking a CDR.

       The invention furthermore provides a method
for the generation of a scFv encoding DNA with a frame-
25 work suitable for selective alterations in the CDR re-
gion, wherein specific restriction sites are introduced
into the sequence of a defined, stable and soluble scFv
encoding DNA by means of site directed mutagenesis
whereby said restriction sites are preferably located
30 within the framework and whereby the substitution of the
nucleotides to generate the restriction site does not af-
fect the amino acid sequence.

       An improved scFv with defined framework that
is stable and soluble in a reducing environment can also
35 be obtained by a method that is also an object of the
present invention, wherein at least two variations of at
least two different frameworks that are stable and solu-

ble in a reducing environment, preferably frameworks of the present invention are combined to produce a scFv with defined framework.

A scFv obtainable by the above described
5   method is also an object of the present invention. In such framework it is preferred that at least one of the variations is preceding the CDR1 of the variable light chain and/or at least one of the variations is located between CDR2 and CDR3 of the variable heavy chain.

10   In a much preferred embodiment the scFv of the present invention comprises at least 2 variations preceding CDR1 of the variable light chain and at least 2, preferably at least 4 variations located between CDR2 and CDR3 of the variable heavy chain, in particular a
15   scFv comprising the framework defined in SEQ ID NO 1.

In order to specifically randomize the CDRs in such framework, silent changes, still coding for the same amino acid sequences but using different codons, can be introduced which lead to the generation of unique re-
20   striction sites (see also above). While the restriction sites can be located anywhere in the CDR/framework-connecting regions, it is preferred if they are located in the framework flanking each individual CDR. By this, each individual CDR can be replaced by introducing random
25   or defined sequences. This allows to select for novel CDR in the intrabody showing a high affinity to the antigen.

When additional sequences, like localization signals or activation domains are introduced into a non-defined framework, stemming from a scFv library, it is
30   possible that due to this modifications, the biological activity – even if hitherto present – is lost, e.g. the scFv gets insoluble. Therefore it is of advantage to use a defined framework of the present invention to a known antigen and subsequently introduce such modifications at
35   different locations in the intrabody (N- and C-terminal or within the coding sequence of the scFv) and select for the maintenance of the original function. WO 99/28502 de-

scribes several possibilities to introduce a localization
signal. The activation domain used for interaction
screenings to an antigen has been described in WO
99/98502 to be introduced at the C-terminus of the scFv
5   library. It has now been found that by the method of the
present invention also frameworks can be selected which
accept additional sequences at different locations, e.g.
the activation domain at the N-terminus, which still per-
form similar to their scFv counterparts, having no acti-
10   vation domain, in the antagonistic function. Therefore,
e.g. in the framework further described in the following
examples, introducing the activation domain N-terminal
does not impair the antibody function.

Starting from an intrabody of the present in-
15   vention with a defined framework that is stable and solu-
ble in reducing environment, scFv or intrabodies, respec-
tively, containing CDR libraries can be generated.

A suitable method for the generation of a CDR
library with a defined framework, that is stable and
20   soluble in a reducing environment is a method of the pre-
sent invention, wherein DNA sequences encoding a scFv of
the present invention are digested to replace at least
one CDR per sequence by a modified CDR. Preferably the
modified CDR is generated by random changes. By such
25   method a library of intrabodies with at least one random-
ized CDR and defined framework that is stable and soluble
under reductive conditions can be generated.

The intrabodies of the present invention con-
taining CDR libraries can be used to screen and select
30   for clones having a high affinity to the antigen. Such a
method for screening for CDRs interacting with a specific
antigen is also an object of the present invention and
comprises host cells transformed with a nucleic acid se-
quence, in particular a DNA sequence, encoding a known
35   antigen which are further transformed with a randomized
CDR library with defined framework that is stable and
soluble in a reducing environment, whereby the antigen

and/or the scFv are linked to a marker system or part of
a marker system thus that the cell cultured under selec-
tive conditions only survives in the presence of anti-
gen/scFv-interaction, that thus transformed cells are
5   cultivated under selective conditions, and that surviving
cells are cultured and the intrabodies harvested.

         In a preferred embodiment of the present in-
vention the framework is a framework of the present in-
vention and the cell is an eukaryotic cell, in particular
10 a yeast cell.

         In a much preferred embodiment of the present
invention the DNA sequence encoding the antigen and the
DNA sequence encoding the scFv both encode chimeric mole-
cules with the antigen or scFv, respectively, both linked
15 to part of a transcription activating system linked to a
survival allowing marker, more preferably the antigen is
fused to a DNA binding domain and the scFv is fused to a
transcriptional activator domain or the antigen is fused
to a transcriptional activator domain and the scFv is
20 fused to a DNA binding domain.

         The intrabodies of the present invention con-
taining CDR libraries can be used to screen and select
for clones having a high affinity to the antigen. This
can either be achieved by blocking the intracellularly
25 located antigen in its biological function or by assaying
for direct interaction of the CDRs embedded in the de-
fined framework to the antigen. Direct interaction can,
preferably, be monitored by a transcriptional readout,
preferably by the expression of the HIS3 gene. Adding 3-
30 aminotriazol (3AT) to the medium, allows to select for
higher affinity of the CDRs to the antigen under said
predetermined conditions. Host cells which are able to
express a specific known antigen only survive in the
presence of antigen-scFv-interaction under said condi-
35 tions, preferably in the presence of sufficiently strong
antigen-scFv interaction. The term sufficiently strong as
used herein is defined as protein-protein interactions

having a K_D, measured by BIAcore, which is $> 1x10^{-6}$ M,
preferably a $K_D > 1x10^{-8}$ M and more preferably a $K_D > 1x10^{-10}$ M. Such a selection step can further be applied to per-
form affinity maturation by random or selective changes

5   of amino acids in the CDR (preferably CDR1 and CDR2 of
the light and heavy chain) and subsequently select out of
this pool for growth on increased 3AT concentration.

        As already mentioned above, hitherto known
and used scFv libraries stem from the isolation of mRNA

10   from preferably spleen which is known to have a high ac-
cumulation of B cells and therefore rearranged antibodies
are expressed. Such a library has the drawback that it
has been pre-selected (positive and negative selection)
not to react against epitopes present in this organism.

15   This guarantees that only antibodies can mature and be
activated which do not start an autoimmune reaction. How-
ever, due to this selection steps, not all possible amino
acid combinations are present in such a "natural" scFv
library. For several in vitro and diagnostic applica-

20   tions, antibodies are required interacting with proteins
which are conserved among species. For such proteins or
peptides, it might be very difficult to find strong in-
teracting monoclonal antibodies in "natural" scFv librar-
ies due to the pre-selection steps. Furthermore, the

25   frameworks present in such "natural" libraries are not
optimized, therefore insufficient or variable solubility
and/or stability, respectively, generates problems.
Therefore it is of great advantage to use only CDR random
libraries comprising a framework of and/or obtainable

30   with the method of the present invention and, covering
some or, preferably, all possible combinations of amino
acid sequence in these regions.

        In order to further describe the present in-
vention, a stable and soluble intrabody framework with

35   defined complementary determining regions (CDRs) directed
against a yeast intracellular transcription factor Gcn4p
was selected. This defined framework was used to replace

the CDRs by random sequences. These CDR libraries are screened to identify new CDRs which provoke a demanded biological activity (*in vivo* effect of the CDRs):

a) Molecular interactions which occur natu-
5 rally within the cell (e.g. in human cells or any other heterologous cells) are reconstituted in a suitable cell, preferably yeast, or yeast endogenous interactions are used. A subsequent screening identifies high affinity CDRs due to the interference of these CDRs with the bio-
10 logical activity of the reconstituted or endogenous molecules. Such an antagonistic CDR could e.g. function by blocking two proteins involved in signal transduction pathways.

b) Agonistic CDRs are selected which induce a
15 demanded biological activity on the reconstituted or endogenous molecules.

The random CDRs embedded in the stable framework can further be used to identify interactions of the CDR with an antigen based on interaction screenings:
20 a) It could be shown that the selected framework can be fused to a transcriptional activation domain and still retains its function. This chimeric intrabody is used to select for high affinity CDRs against a given antigen fused to a DNA-binding domain or a transcription
25 factor which possesses DNA-binding activity. Upon interaction of the antigen and the CDRs, the transcriptional activation domain mediates gene expression of a selectable marker gene thus allowing survival of this cell under selective conditions.
30 b) A reconstituted molecular interaction based on hybrid technique (fusion of one partner to activation domain, the other if necessary to DNA-binding domain) can be blocked by specific, high affinity CDRs.

It was also found that different mutations in
35 the framework but constant CDRs of the intrabody have an effect on its *in vivo* performance by changing the stability and solubility of the intrabody. The framework con-

tributes the major part to the stability and solubility
of an intrabody. Nevertheless, certain mutations in the
CDRs might also affect solubility and stability of the
intrabody. Therefore it might be advantageous to pre-
5   select the random CDRs embedded in a defined framework by
a functional quality control (see below).

           The present invention furthermore provides a
method for the identification of intrabody frameworks or
intrabodies wherein suitable host cells are transformed
10   with a library and a marker system, whereby said library
is a fusion product of an intrabody library and at least
part of said marker system, wherein said marker system is
only activated in the presence of a fusion protein encod-
ing a soluble and stable intrabody framework, and cultur-
15   ing said cells under conditions allowing the identifica-
tion and selection of cells expressing a soluble and sta-
ble intrabody framework.

           In a preferred embodiment of the present in-
vention said library is a fusion product of an intrabody
20   library and a marker protein. Preferably said marker pro-
tein has a selectable activity, in particular an enzy-
matic activity or fluorescence activity. A marker protein
that can be used in such a method is e.g. the GFP protein
or any mutant thereof.

25   In another preferred embodiment of the pres-
ent invention said library is a fusion product of an in-
trabody library and a DNA binding protein that can acti-
vate transcription of a marker gene whose transcription
is under control of said DNA binding protein. A suitable
30   DNA binding protein is e.g. p53.

           In a further preferred embodiment of the pre-
sent invention said method comprises suitable host cells
that are transformed with a library that encodes proteins
comprising an intrabody and one part of a transactivation
35   system and said cells further express a second protein
comprising at least the second part of said transactiva-
tion system, whereby said transactivation system is

linked to a survival allowing marker and said cells only
survive under selective conditions in the presence of an
interaction between said two fusion proteins.

In a more preferred embodiment said library
5   encoded proteins comprise a transcriptional activation
domain and said second proteins comprise a DNA binding
domain or said library encoded proteins comprise a DNA
binding domain and said second proteins comprise a tran-
scriptional activation domain.

10   In a further preferred embodiment said second
proteins comprise a DNA binding domain or a transactiva-
tion domain, respectively, and a protein interacting with
a constant region of said library encoded protein. The
term constant region as used herein encompasses any pro-
15   tein domain or any contiguous stretch of amino acids that
is encoded by the library construct and can serve as pro-
tein interacting partner and said term includes e.g.
parts of the intrabody or Ga111p.

An scFv with defined framework obtainable by
20   the above methods is also an object of the present inven-
tion, in particular for the use in a method of the pres-
ent invention.

The same methods can also be applied for the
screening of any scFv library to identify soluble and
25   stable frameworks that may e.g. be used as starting mate-
rial for a scFv or CDR library, in particular libraries
of the present invention.

Another object of the present invention is to
provide a method for screening for an antigen interacting
30   with an scFv, wherein host cells expressing at least one
antigen of interest are transformed with at least one
scFv with defined framework that is stable and soluble in
reducing environment, or with a randomized CDR library
with defined framework that is stable and soluble in re-
35   ducing environment, whereby the antigens and/or the scFvs
are linked to a marker system or part of a marker system
thus that the cell cultured under selective conditions

only survives in the presence of antigen/scFv-
interaction, that thus transformed cells are cultivated
under selective conditions, and that surviving cells are
cultured and the scFvs harvested.

5   In a preferred embodiment of the present in-
vention the framework is a framework of the present in-
vention and the cell is an eukaryotic cell, in particular
a yeast cell.

           In a much preferred embodiment of the present
10   invention the DNA sequence encoding the antigen and the
DNA sequence encoding the scFv both encode chimeric mole-
cules with the antigen or scFv, respectively, both linked
to part of a transcription activating system linked to a
survival allowing marker, more preferably the antigen is
15   fused to a DNA binding domain and the scFv is fused to a
transcriptional activator domain or the antigen is fused
to a transcriptional activator domain and the scFv is
fused to a DNA binding domain.

           The invention furthermore provides an scFv
20   with defined framework as therapeutic or diagnostic or
prophylactic agent and the use of the scFv with defined
framework for intracellular screenings.

           For all purposes of the present invention
eukaryotic cells are preferred, whereby yeast cells are
25   especially preferred due to their specific features in-
cluding e.g. fast growth, positive selection, growth se-
lection and efficient transformation and selection
thereof.

30   Brief Description of the Drawings
           Figure 1A shows how a quality control of the
scFv or CDR library may be performed.

           Figure 1B shows that solubility of the scFv
fusion proteins correlates with reporter gene activation.
35   **Figure 2** shows the better in vivo performance
of the optimized Gal4 AD-Ω-graft scFv compared to another
variant called λ-graft.

       Figure 3A shows *in vivo* performance of different scFv fragments on gene expression of a Gcn4p dependent LacZ reporter gene.

       Figure 3B shows in vivo performance of different scFv fragments expressed in yeast, in a two hybrid assay.

       Figure 4 shows growth selection in a two hybrid assay of cells expressing different scFv fragments.

       Figure 5A shows that the N-terminal fusion of a constant domain (Gal11P-Gal4AD) to a single chain antibody does not significantly change the property of this scFv fragment on gene expression of a Gcn4p dependent LacZ reporter

       Figure 5B shows that the introduction of two unique restriction site in a single chain antibody does not change the property of this scFv fragment on gene expression of a LacZ reporter.

       Figure 6 shows western blot analysis of solubility of different Gcn4p binding scFv fragments expressed in yeast.

Modes for Carrying out the Invention

Quality control of the scFv and CDR libraries

       The term "quality control" defines an assay that allows the selection of a stable and soluble intrabody from a scFv library.

       For this purpose a fusion of the scFv library to a transcriptional activation domain (in this case Gal4AD) and a constant region (in this case Gal11P aa 263-352) is generated. Stability of the fusion protein depends on the stability and the solubility of the scFv portion. The constant Gal11P domain interacts with the dimerization domain of Gal4 (residues 58-97, part of the Gal 4 DNA-binding domain (DBD) (Barberis et al., 1995)).

       This library is transformed into a yeast cell expressing the Gal4 DBD (residues 1-100) which binds to

the promoter of a selectable marker gene (e.g.
HIS3/LacZ). Growth of this host cell is only mediated
when the tested intrabody shows the demanded solubility
and stability and therefore can sufficiently interact via
5 Gal11P with the Gal4 DBD (see Figure 1A).

Solubility correlates with gene activation

The principle of the quality control system
as described in the present invention was demonstrated
10 using a number of well characterised scFvs. These possess
essentially identical antigen binding properties but dif-
ferent *in vitro* stabilities. The different scFv fragments
were expressed as Gal11P-Gal4AD fusion proteins. The Gal4
dimerization domain (residues 58-97) was fused to the C-
15 terminus of LexA and transformed into the reporter
strain YDE173, containing reporter genes under the con-
trol of 6x LexA binding sites (see below).

As stated above, the intracellular stability
and solubility of the Gal11P-Gal4AD-scFv fusion proteins
20 depends on the scFv portion. Therefore, only stable and
soluble scFv fusion proteins interacting sufficiently
with LexA-Gal4(58-97) are able to activate reporter gene
expression (e.g. β-galactosidase)

The wt allele of Gal11 does not interact with
25 the Gal4 dimerization domain (residues 58-97). A fusion
of any single chain with the Gal11 wt allele is therefore
unable to activate the reporter gene and serves as a
negative control. This was demonstrated using a Gal11wt-
Gal4AD-λ graft fusion construct (see Figure 1B).
30 Neither the bait (LexA-Gal4(58-97)) nor the
scFv fusion protein alone activate reporter gene expres-
sion.

Only two out of six tested scFv fragments
were soluble and stable enough to activate reporter gene
35 expression in our quality control system. The framework
stabilized λ- graft and the κ-graft are the most stable
variants. This result correlates perfectly with frac-

tionation analysis, where only the λ- and κ-graft were found in the soluble fraction. (see Figure 6).

5   ***ScFv fragments cytoplasmically expressed in yeast***

Suitable scFv fragments are e.g. the anti-GCN4 wild-type scFv that has originally been obtained by ribosome display from a library constructed from an immu-
10   nized mouse (Hanes *et al.*, 1998). The antigen was a double proline mutant of the Gcn4p leucine zipper, called 7P14P (indicating that positions 7 and 14 of the zipper domain are mutated to Pro residues), which forms a random coil in solution (Leder *et al.*, 1995). The scFv fragment
15   prevents dimerization of the wild-type Gcn4p coiled coil peptide *in vitro* (Berger *et al.*, 1999), as it also binds the wild-type peptide as a monomer in a random coil conformation. The anti-GCN4 scFv fragment referred to as "wild-type" in connection with the present invention has
20   been measured to have a dissociation constant of $4 \cdot 10^{-11}$M from the leuzine zipper peptide (Hanes *et al.*, 1998).

In the scope of the present invention, several different mutants of this scFv were investigated. Besides the anti-GCN4 wild-type, a destabilized variant
25   of the anti-GCN4 wild-type, which carries the H-R66K mutation [termed anti-GCN4(H-R66K)], served as an example for a Gcn4p binding scFv fragment with essentially identical antigen binding properties, but with slightly decreased *in vitro* stability (see below). The Arg residue
30   at position H-66 (numbering according to Kabat *et al.*, 1991) is far away from the antigen binding pocket and usually forms a double hydrogen bond to Asp H-86. Arg at position H-66 was shown previously to result in higher protein stability than a Lys in the levan binding A48
35   scFv fragment (Proba *et al.*, 1998; Wörn and Plückthun, 1998a). Moreover, a Val-Ala variant of the anti-GCN4 scFv fragment [termed anti-GCN4(SS⁻⁻)] was tested, where both

intradomain disulfides were replaced by Val-Ala pairs (L-
C23V, L-C88A, H-C22V, H-C92A). These mutations had been
shown to act slightly stabilizing compared with the re-
duced dithiol form of the p185HER2 binding 4D5 scFv frag-

5   ment before, and it had been speculated that they might
improve the performance of intrabodies (Wörn and Plück-
thun, 1998b).

Two additional variants were engineered by
grafting (Jones *et al.*, 1986) the anti-GCN4 CDR (comple-

10 mentarity determining region) loops to another framework.
As the acceptor framework the so-called "hybrid" scFv was
chosen (Wörn and Plückthun, 1999). This acceptor framework
is composed of the V_L domain of the 4D5 scFv fragment and
the V_H domain of the A48[++](H2) scFv fragment. It had been

15 rationally designed from a series of stabilized domains
and stands out for its extraordinary stability, as demon-
strated by denaturant induced equilibrium unfolding, and
a high expression yield (Wörn and Plückthun, 1999). Two
CDR-grafted variants with the anti-GCN4 scFv CDRs and the

20 "hybrid" scFv framework were prepared by total gene syn-
thesis. As the anti-GCN4 wild-type loop donor carried a λ
light chain, while the acceptor "hybrid" framework car-
ried a κ light chain, the loop grafting was not straight-
forward. Therefore, two different variants were designed,

25 one more "κ-like" (termed κ-graft), the other more "λ-
like" (termed λ-graft). These two variants differ only in
seven residues in the V_H-V_L interface region, potentially
influencing the orientation of the two domains to each
other. The ampicillin-binding scFv fragment AL5 (A. Kreb-

30 ber *et al.*, unpublished) served as a negative control for
a scFv fragment not binding Gcn4p.

Anti-GCN4 scFv intrabodies inhibit the trans-
35 ***activation potential of Gcn4p***

The anti-GCN4 scFv was initially tested for
its biological activity expressed from several yeast vec-

tors including *GAL1* and *ADH*-driven promoters. In addition, the nuclear localization signal (NLS) from SV40 large T-antigen was fused N-terminally to the anti-GCN4 scFv. Of the combinations tested, the anti-GCN4 scFv

5   showed the strongest biological effect when expressed from the actin-1 promoter without any NLS using the pESBA-Act expression vector (see Examples) with *TRP1* selection marker and 2μ origin (data not shown). This vector was subsequently used for all further experiments.

10   The *in vivo* effect of expressing the different scFv fragments on *GCN4* dependent *LacZ* expression is depicted in Figure 3A. The reporter construct (YAdM2xGCN4-150) contained two Gcn4p binding sites at position -150 relative to the TATA box and was integrated

15   into the yeast genome. Relative β-galactosidase activity (*Rel. β-gal. activity*) driven by endogenous Gcn4p was arbitrarily set to 100%. AL5 is an ampicillin binding scFv fragment and serves as negative control. Besides the anti-GCN4 wild-type (wt), a destabilized point mutant

20   [anti-GCN4(H-R66K)], a cysteine-free variant of the anti-GCN4 wild-type [anti-GCN4(SS^{--})], and two framework stabilized variants of anti-GCN4 (κ-graft and λ-graft) were tested. The stabilized λ-graft was the most active intrabody, whilst the destabilized H-R66K point mutant and

25   the cysteine-free variant of anti-GCN4 showed decreased activity, compared to the anti-GCN4 wild-type. The decreased activity of the κ-graft is believed to be due to its low binding affinity (see Table 1). The destabilized point mutant anti-GCN4 (H-R66K) was less efficient in in-

30   hibition of *GCN4* dependent reporter gene activity, compared to the wild-type scFv. The pattern of Gcn4p transactivation inhibition was highly reproducible and was also confirmed when using a different assay method, where β-galactosidase reporter activity was measured after dis-

35   rupting the cells by glass beads or freeze-thaw cycles for lysis and normalizing the β-galactosidase activity to protein concentration (Escher and Schaffner, 1997) (data

not shown).

Table 1

Protein	K_D [M]	measured β-galactosidase activity (%)	approximate onset of denaturation ([M]
anti-GCN4 wt	$4.36 \pm 0.09 \cdot 10^{-11}$	52 ± 1.38	1.7
anti-GCN4 (H-R66K)	$4.21 \pm 2.66 \cdot 10^{-11}$	66 ± 1.98	1.4
λ-graft	$3.80 \pm 0.76 \cdot 10^{-10}$	16 ± 0.50	2.0
κ-graft	$1.57 \pm 0.14 \cdot 10^{-06}$	79 ± 2.37	2.6

The Gal4 AD-scFv fusion proteins perform in a two hybrid assay according to their in vitro stability and in vivo performance.

The successful interaction between the antigen and the complementary determining regions (CDRs) in the two hybrid assay monitoring LacZ expression as a reporter gene is shown in Figure 3B. The reporter strain YDE173 was used. Strain YDE173 was deposited on February 11, 2000 with the Deutsche Sammlung von Mikroorganismen und Zellkulturen DSZM, Braunschweig Germany, under the Number DSM 13333. YDE173 was derived from yeast strain JPY5 (Matα ura3-52 his3Δ200 leu2Δ1 trp1Δ63 lys2Δ385) having integrated at the genomic his3 locus the reporter plasmid pDE200 which contains six LexA binding sites controlling the divergently oriented reporter genes HIS3 and LacZ.
The same scFv fragments as used for Fig. 3A, but fused to the transcriptional activation domain of Gal4 were coexpressed together with the GCN4 leucine zipper (aa 245-285) fused C-terminal to LexA, serving as a bait for the two hybrid assay. The unspecific AL5 control scFv fusion construct was unable to interact with the LexA-GCN4 leu-

cin zipper and therefore did not activate the LacZ re-
porter gene. The Gal4 activation domain fused to the
framework stabilized λ-graft variant exhibited the
strongest effect as activating intrabody, followed by the
anti-GCN4 wild-type, and the destabilized point mutant
anti-GCN4 (H-R66K). In contrast the highly stable but
weakly binding κ-graft and the cysteine-free anti-GCN4
(SS⁻⁻) caused no significant reporter gene expression in
the two hybrid format. The same results were obtained in
an X-Gal plate assay (data not shown). In summary, the *in
vivo* performance of the different Gal4 AD-scFv fusion
variants in activating the LacZ reporter gene in the two
hybrid format correlates reciprocally to the inhibition
pattern of the Gcn4p dependent LacZ expression (compare
Figure 3A with 3B).

***Interaction between the antigen and the dif-
ferent scFv's fused to a transcriptional activation do-
main allows growth selection in a two hybrid assay***

Since the integrated reporter construct con-
tains not only a LacZ reporter gene but also the HIS3
gene, it is suitable for growth selection on plates lack-
ing any histidine. Furthermore, by adding different con-
centration of 3-aminotriazol (3-AT), which is a competi-
tive inhibitor of the HIS3 gene product, it is possible
to inhibit (suppress) growth of the yeast cells dependent
on the strength of the interaction between bait/antigen
and Gal4 AD-scFv.

The experimental procedure leading to the re-
sults shown in Figure 4 was as follows: A serial 5-fold
dilution, starting with approximately 10´000 yeast cells
coexpressing the GCN4 leucine zipper (aa 245-285) fused
to LexA and a Gal4-AD scFv fusion protein, were spotted
on drop out plates (-Trp/-Leu/-His) containing different
concentrations of 3-AT. Growth was monitored after 48h,
72h, and 120h.

The lanes in Figure 4 are as follows:

1. Gal4-AD λ-graft, 2. Gal4-AD AL5, 3. Gal4-AD κ-graft, 4. Gal4-AD anti-GCN4 (SS⁻⁻), 5. Gal4-AD anti-GCN4 wild-type, 6. Gal4-AD Anti-GCN4 (H-R66K), 7. LexA-Gal11 fusion protein serves as positive control, 8. empty vectors.

Growth of the yeast strains coexpressing the bait/antigen (lexA-GCN4 leucine zipper) together with a Gal4 AD-scFv fusions was monitored over five days. As a control on plates lacking 3-AT, no obvious growth difference of the different Gal4 AD-scFv fusion variants was observed. Already 20 mM 3-AT were enough to suppress growth of the cells transformed with the negative control scFv (Gal4 AD-AL5). In correlation with the results monitoring β-galactosidase expression, the Gal4 AD fusions with the κ-graft variant, anti-GCN4 (SS⁻⁻), and anti-GCN4 (H-R66K) did not allow growth in the presence of 20 mM 3-AT. Cells expressing the λ-graft variant as well as the anti-GCN4 wild-type were able to grow in the presence of up to 80 mM 3-AT within 5 days with a clear advantage for the framework stabilized λ-graft over the time. A concentration of 100 mM 3-AT was enough to abolish growth of cells expressing Gal4 AD-anti-GCN4 wild-type. Only after five days, a few appeared on the most concentrated spotting whereas cells expressing the λ-graft Gal4 AD-scFv fusion variant clearly grew.

The N-terminal fusion of constant domain(s) to the λ-graft scFv does not interfere with its biological activity

Gal11P (residues 263-352) and the Gal4 activation domain was fused to the N-terminus of the λ-graft scFv (Gal11P-Gal4AD λ-graft). Its biological activity in inhibiting the Gcn4p dependent gene activation was compared to λ-graft alone. As shown in Figure 5A the fusion of a constant domain to the scFv did not interfere with the inhibitory activity on Gcn4p dependent gene activation.

Introduction of specific restriction sites

In order to exchange the CDR3 V_H (GLFDY)(Seq. Id. No. 2) with a random peptide library, two unique re-

5   striction sites (*Bgl*II and *Xho*I) flanking this hypervariable region were introduced by silent mutagenesis. These silent changes did not affect the amino acid sequence of the antibody and therefore did not alter the in vivo performance of the λ-graft variant (see Figure 5B).

10   The importance of the CDR3 hypervariable region (de Wildt *et al.*, 1997; Hemminki *et al.*, 1998) for specific recognition of its antigen (GCN4 leucine zipper) was shown by introducing an additional alanine N-terminal to the CDR3 (**A**GLFDY) (Seq. Id. No. 3) of the variable

15   heavy chain. This λ-graft+Ala variant failed to inhibit expression of a Gcn4p dependent reporter gene in the yeast strain YAdM 2xGCN4-150, and was also unable to activate reporter gene expression in the two hybrid format using the strain YDE173 (data not shown).

20

Both graft variants are soluble in yeast cytoplasm

The solubility of the different Gcn4p binding scFv fragments in yeast was tested by Western blot analy-

25   sis. Only in case of the λ- and κ-graft variants significant amounts of soluble protein could be detected in crude cell extracts (Figure 6).

All other anti-GCN4 scFv fragments appeared to be essentially completely insoluble, with the amount

30   of insoluble scFv slightly increasing with decreasing *in vitro* stability. However, one has to caution that the exact ratio of soluble to insoluble protein for the different scFv variants may not necessarily reflect the ratio present *in vivo*. It cannot be excluded that part of the

35   different anti-GCN4 variants might have precipitated during sample preparation, even though we used a gentle cell disruption method, by using the Y-PER™ Yeast Protein Ex-

traction Reagent form Pierce.

Improvement of the framework

Variations in frameworks preferably isolated
by a method according to the present invention can be
combined to generate further frameworks that are stable
and soluble in a reducing environment. Said resulting
frameworks show an enhanced in vivo performance compared
to frameworks bearing only one variation. A framework
combining six variations is defined in SEQ ID NO:1.

Examples

Design of CDR-grafted anti-GCN4 scFv frag-ments

Cloning, expression and purification of scFv fragments

All scFv fragments were in a V_L-V_H orientation
with a 20-mer linker (GGGGSGGGGSGGGGSSGGGS) (Seq. Id. No.
4) and a C-terminal his_5-tag.

The scFv fragments expressed in yeast were
cloned into the pESBA-Act expression vector. The pESBA-
Act vector is a Saccharomyces cerevisiae - E.coli shuttle
vector. It contains a bacterial origin of replication and
the amp resistance gene. Furthermore it contains the
yeast TRP1 gene for transformation selection in S. cere-
visiae. It is designed for high protein expression in
yeast and therefore has the 2μ origin of replication en-
suring high copy numbers in S.cerevisiae. In addition, it
contains the strong constitutive actin promoter and the
GAL11 transcriptional termination sequence separated by a
multiple cloning site containing restriction sites for
NcoI (covering translational initiation codon ATG), ApaI,
StuI, three translational stop codons in all three frames
and a SalI site.

All scFv fragments were cloned via *Bsp120I*
and *StuI* restriction sites and carried a C-terminal His_5-

tag. Two amino acids (Gly-Pro) encoding the *Bsp120I* site had to be included at the N-terminus, after the initiating Met residue.

5   ***Fusion of the Gal4 AD N-terminal to the various antibody variants.***

The Gal4 activation domain was amplified by polymerase chain reaction using pGAD424 (Clontech) as template. Both primers [upstream primer:

10   5'-CCATGGGCCCAAGCTTTGCAAAGATGGATAAAG-3' (Seq. Id. No. 5, downstream primer: 5'-TTTGGGCCCGAAGAACCGCCACCACCAGAACCG CCTCCACCAGAGCCACCACCACCAGGCCTGATCTCTTTTTTTGGGTTTGGTG-3', (Seq. Id. No. 6)] contain an *ApaI* site suitable for cloning the Gal4 activation domain (AD) polypeptide including

15   the SV40 T-antigen nuclear localisation signal N-terminal to the different scFv's in the context of pESBA Act. The activation domain and the single chain antibodies are seperated by a (GGGS)₃ linker (Seq. Id. No. 7) encoded by the downstream primer.

20

N-terminal fusion of Gal11wt and Gal11P to the Gal4 activation domain (AD)-scFv fusion

Gal11wt and Gal11p were both amplified using the following primers: upstream primer: 5'-
25   CATGCCATGGTTCCTCAACAGCAGCAAATGCAAC-3' (Seq. Id. No. 8), downstream primer: 5'-CATGCCATGGCGCTAGCCAAAGCTTGGATTTTT CTCAGG-3' (Seq. Id. No. 9), both containing an *NcoI* site. The PCR products encoding amino acids 263-352 were inserted into the *NcoI* site of the pESBA-Act2 Gal4(AD)-scFv
30   fusion construct (described above). This generated an in frame fusion of the respective Gal11 allele with Gal4(AD)-scFv. Correct orientation of the Gal11 inserts was checked by digestion with the unique enzyme *NheI*.

35   ***LexA fusion***

The GCN4 leucine zipper (aa 245-285) was PCR amplified with primers containing an *EcoRI* site conven-

ient for cloning downstream of LexA aa 1-202. This re-
sults in pAdM018, an Ars Cen plasmid with the LEU2 selec-
tion marker expressing the fusion protein under the con-
trol of the ADH promoter.

5

Introduction of a BglII and XhoI site flank-
ing CDR3 of V$_H$

In order to easily exchange the CDR3 of the
variable heavy chain, two unique restriction sites were
10   introduced flanking the CDR3 V$_H$ by site directed muta-
genesis, without changing the primary structure of the
Gal4 AD-λ-graft scFv. These silent point mutations were
introduced by PCR using λ-graft as template. In a first
round, two seperate PCR reactions were performed using
15   primer #2421 with #2487 and #2486 with #2488 leading to
two overlapping PCR products. These two products served
as template for the second round of PCR with the outer
primer #2421 and #2488 containing a SpeI and SalI site.
The final product was subcloned into Gal4 AD-λ-graft us-
20   ing SpeI and SalI.

Direct intracellular screening for novel CDRs
interacting with the antigen.

The first three amino acids (GLF) of the CDR3
25   from the variable heavy chain of the framework stabilized
λ-graft scFv fused to the Gal4 activation domain (λ-graft
scFv-Gal4 AD) were randomized with a PCR based method de-
scribed by Reiter et al. The last two residues (D and Y)
of the CDR3 were not randomized due to their conservation
30   and structural importance (Chothia and Lesk, 1987). A λ-
graft scFv-Gal4 AD library potentially encoding 8000 dif-
ferent CDR3 variants of the variable heavy chain was ob-
tained. Sequence analysis of six randomly picked library
clones revealed the presence of random CDR3 sequences at
35   the expected positions.

The yeast strain YDE173, containing the HIS3
and LacZ reporter genes under the control of 6 LexA bind-

ing sites (see above), was cotransformed with the vector
expressing the GCN4 leucine zipper (aa 245-285) fused to
LexA and the library and plated on selective drop out
plates (-Trp/ -Leu/ -His) containing 60 mM 3-AT for
5   growth selection. If a scFv fragment from the CDR3 li-
brary with a suitable CDR3 sequence binds to the leucine
zipper antigen fused to LexA, a complex is formed that
activates transcription of the *HIS3* reporter gene and re-
stores histidine independent growth of the yeast cell.
10   After 3 days, growing colonies were picked and replated
on the same selective drop out plates. Cells that still
grew after the second selection were analyzed for β-
galactosidase activity on X-gal plates. Library plasmid
DNA from β-gal positive clones was extracted and the re-
15   gion of the CDR3 of the variable heavy chain was se-
quenced: We found four times the original λ-graft CDR3
amino acid sequence and 3 completely new CDR3 sequences
specific for the GCN4 leucine zipper. The four identified
scFv clones containing the original CDR3 sequence behaved
20   indistinguishable as the λ-graft whereas the three clones
with the altered CDR3 sequence were less efficient in ac-
tivating the LacZ reporter gene.

      These results demonstrate the feasibility of
a direct intracellular screening for novel CDRs embedded
25   in a defined scFv framework that is stable and soluble
under reducing conditions.

      **In vivo performance of a defined intrabody
can be optimized by random mutagenesis**
30   The framework stabilized λ-graft variant was
randomly mutagenized by PCR as described by Sambrook et
al. in order to statistically introduce amino acid
changes along the framework of the intrabody. The yeast
strain YDE173 was cotransformed with this random muta-
35   genized scFv library fused to the activation domain of
Gal4 and the plasmid expressing the specific antigen (aa
245-258 of the GCN4 leucine zipper) fused to LexA and

grown on drop out plates containing 80 mM 3-AT. Six can-
didate clones were selected, each bearing one single
amino acid change in the framework. All these six mutant
frameworks showed an improved in vivo performance com-
5   pared to the λ-graft variant, which was confirmed and
quantitated by measuring the β-galactosidase activity.
With the assumption that different amino acid changes
which improve the performance of an intrabody behave ad-
ditively, we combined all six mutations in one framework
10 which was fused to the Gal4 activation domain and com-
pared it with the framework stabilized λ-graft variant in
activating the LacZ reporter gene. Figure 2 shows that
this new framework which has all six point mutations com-
bined (Ω-graft) displays an almost 30% better in vivo
15 performance compared to the original λ-graft variant. Re-
markably, these six amino acid substitutions are clus-
tered; two of them (E→K and L→R are preceding the CDR1
of the variable light chain and the remaining four (N→D,
G→C, K→E,T→S) are located between CDR2 and CDR3 of the
20 variable heavy chain.

**Integration of a reporter gene into the chro-
mosome of Saccharomyces cerevisiae**

25   The integrating reporter plasmid pAB183 was
derived from pJP161 (Barberis *et al.*, 1995) by cloning
two Gcn4p binding sites at position 150 upstream of the
TATA box of the GAL1 promoter. The Gcn4p binding sites
were generated by annealing two complementary oligonu-
30 cleotides having a 5' *Sph*I and 3' *Sal*I compatible over-
hang sequence. The oligonucleotides are as follows:
5'-CCTATGACTCATCCAGTTATGACTCATCG-3' (Seq. Id. No. 10);
5' TCGACGATGAGTCATAACTGGAT GAGTCATAGGCATG-3' (Seq. Id.
No. 11). This reporter plasmid was linearized at the *Apa*I
35 site and integrated into the yeast genomic ura3 locus of
strain JPY5 (Barberis *et al.*, 1995), resulting in
YAdM2x*GCN4*-150. Strain YAdM2xGCN4-150 was deposited on

February 11, 2000 with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH DSZM, Braunschweig Germany, with the Number DSM13332. Four independent yeast transformants were tested in a functional assay and all
5   showed the same *GCN4*-dependent reporter gene activity. One of the clones (YAdM2x*GCN4*-150) was chosen for all subsequent experiments and is called yeast wild-type.

The reporter strain used for the two hybrid experiments, has a integrated reporter construct contain-
10 ing a bidirectional promoter with six LexA binding sites driving LacZ and HIS3 expression.

Serial dilution and spotting of yeast cells

Yeast cells were transformed using the lith-
15 ium acetate method, following standard protocols. Transformants were grown over night at 30°C in drop-out medium (-Trp/-Leu). The saturated cultures were diluted in drop-out medium to OD_{600} = 0.7 and incubated again for at least one duplication time. Each culture was serially diluted
20 in water (dilution factor 5) starting with an approximate concentration of 10^6 cells/ml, and 10 µl of each dilution were spotted on drop-out plates (-Trp/-Leu/-His) containing 0 mM, 20 mM, 40 mM, 60 mM, 80 mM, or 100 mM of 3-aminotriazole. Six different dilutions of each transfor-
25 mant were spotted on drop-out plates. The plates were incubated at 30°C and scanned after 48h, 72h, and 120h.

In vivo analysis of scFv fragments: Expression of scFv fragments in yeast and the β-galactosidase
30 reporter assay

The β-galactosidase assay in solution was performed using permeabilized cells as described (Kaiser *et al.*, 1994, Escher and Schaffner 1997). Activity was normalized to the number of cells assayed.

35

Western blot analysis of anti-GCN4 scFv frag-

ments

The solubility of the different anti-GCN4 scFv fragments was analyzed by Western blot. Five ml cultures were grown at 30°C to an optical density of about

5   2-3. Cells were normalized to same cell densities, pelleted and whole cell protein was extracted with Y-PER™ Yeast Protein Extraction Reagent form Pierce, which is a mild detergent formulation facilitating gentle isolation of soluble proteins. Soluble and insoluble fractions were

10 separated by centrifugation (13000 rpm, 10 min, 4°C). Samples of soluble and insoluble crude extract were subjected to SDS-PAGE and blotted on PVDF membranes, following standard protocols. His_5-tagged scFv fragments were detected with anti-His_5 scFv-AP fusion as described

15 (Lindner *et al.*, 1997), with the chemoluminescent phosphatase substrate CSPD from Boehringer Mannheim. To obtain reasonable intensities on the Western blots, about 5 times higher protein concentrations had to be used in the soluble fractions, compared with the insoluble fractions

20 and the blots were exposed for different time spans. Thus, a direct comparison is only meaningful between all soluble or all insoluble samples, respectively.

While there are shown and described presently preferred embodiments of the invention, it is to be dis-

25 tinctly understood that the invention is not limited thereto but may be otherwise variously embodied and practiced within the scope of the following claims.

32

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